Reprinted from JOURNAL OF INVERTEBRATE PATHOLOGY, Volume 22, Number 1, July 1973

348

JOURNAL OF INVERTEBRATE PATHOLOGY 22, 23–33 (1973)

Observations on the Process of Wound Repair in Penaeid Shrimp[1]

C. T. FONTAINE AND D. V. LIGHTNER

National Marine Fisheries Service Gulf Coastal Fisheries Center, Galveston Laboratory, Galveston, Texas 77550

Received November 6, 1972

The Petersen disk tag is a standard mark for penaeid shrimp, and attachment of the tag involves the insertion of a stainless steel pin through the shrimp's abdomen, resulting in a relatively large puncture wound. The wound healing process first observed at 24 hr post-tagging showed a pronounced hemocytic infiltration of the wound area. Hemocytes in contact with the pin became fusiform, began adhering to one another, and formed several concentric layers around the pin. Scattered foci of bacteria or nercrotic tissue in the vicinity of the wound also became encapsulated by concentric layers of fusiform hemocytes, thereby forming nodules. Melanin appeared in association with the layers of hemocytes nearest the pin and in the nodules. Hemocytic infiltration was followed by the appearance of fibrocytes and the deposition of collagenlike fibers along the wound channel 48 hr after wounding. Involution of epidermis and consequential cuticular involution into the wound channel began at 96 hr after wounding. Complete epidermal and cuticular formation along the wound occurred by 384 hr post-tagging.

INTRODUCTION

Although the Petersen disk tag is the standard mark used in shrimp mark-recapture studies, Lindner and Anderson (1956) and Neal (1969) recognized that the puncture wound made by insertion of the pin caused mortalities, presumably from improper healing of the cuticle and subsequent secondary infection. The wound repair phase of the inflammatory response, though well understood in a number of invertebrates, has been only superficially investigated in the commercially important penaeid shrimp. In the one published account (Fontaine, 1971), gross observations showed that the pin became surrounded by a chitinous tubular exoskeletal-like structure that grew inward from the body surface, relegating the pin to an external rather than an internal position. This paper presents the histopathological aspects of the response to injury of brown shrimp, *Penaeus aztecus*, wounded with the Petersen disk tag pin.

MATERIALS AND METHODS

Animal-collection and maintenance. The experimental shrimp were maintained at a temperature of 28°C and a salinity of 25‰ and were wounded by inserting a stainless steel pin through the musculature between the 1st and 2nd abdominal segments. A small plastic disk was affixed to either side of the shrimp and the pointed end of the pin was clipped to remove the excess and flattened to prevent the tag from being lost (Fig. 1); no antibiotic was used with this procedure. Samples were taken at 24-hr intervals post-tagging for a period of 16 days and were fixed in 10% formalin buffered with sodium acetate. The tags were removed 24 hr following fixation and

[1] Contribution No. 348, National Marine Fisheries Service Gulf Coastal Fisheries Center, Galveston Laboratory, Galveston, Texas 77550.

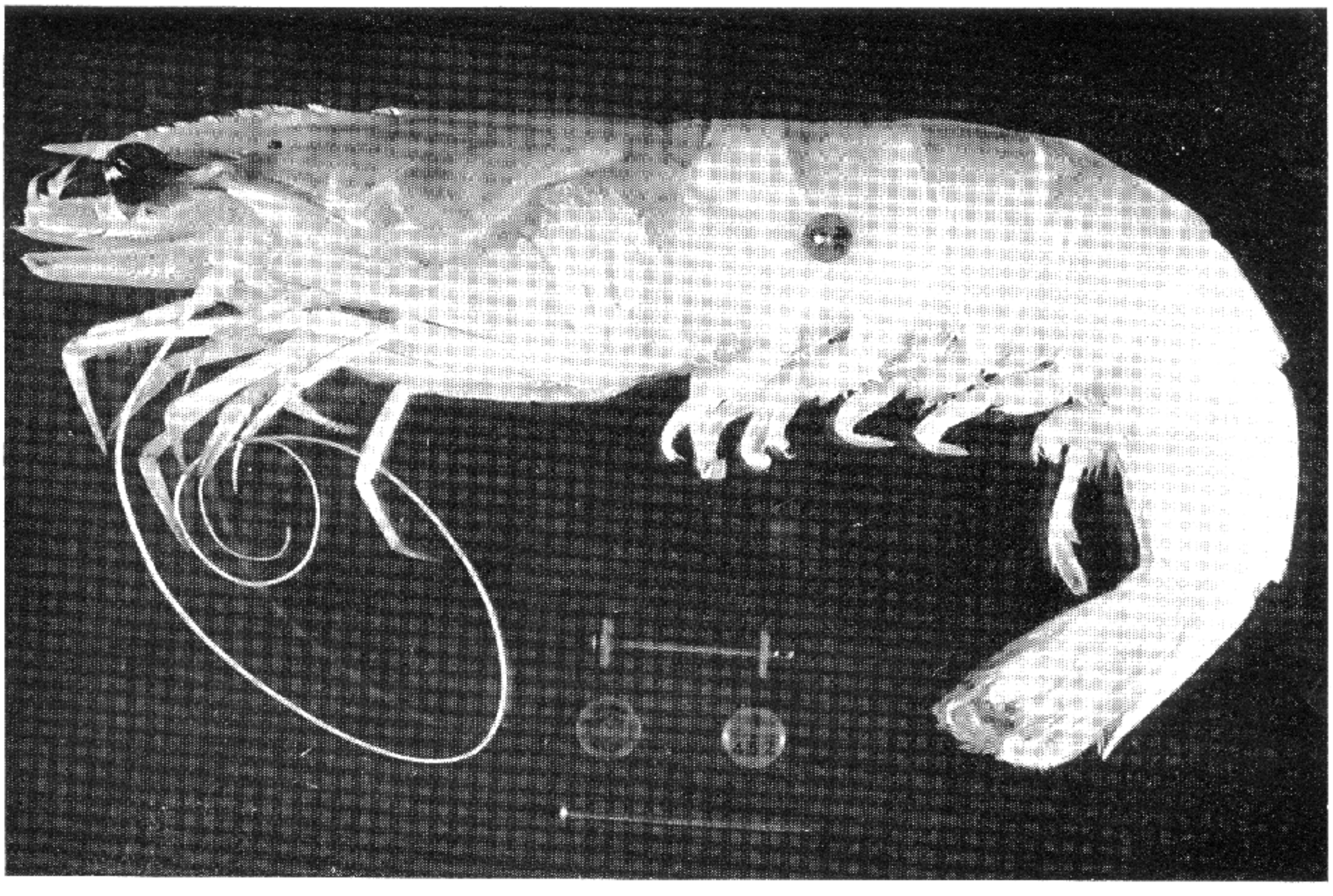

FIG. 1. The Petersen disk tag, and location of pin insertion in the brown shrimp, *Penaeus azlecus*.

the affected tissue was placed in fresh fixative for storage.

Histological procedures. The specimens were decalcified for 8 days utilizing a method modified from the formic acid method of Evans and Krajian (Krajian, 1940). Sodium acetate was used rather than sodium citrate as a buffer. The solution was changed daily, and following decalcification, tissues were washed in tap water for 4–6 hr. The tissues were then dehydrated through a graded ethanol series, cleared in chloroform, and embedded in paraffin. Ten micrometer serial sections were prepared and stained with Harris' hematoxylin. Selected tissue sections from specimens taken at 192 hr were stained with Mallory's triple stain.

RESULTS

Gross Appearance

Within 48 hr post-tagging a white "column," visible through the exoskeleton, had formed around the pin. This "column" assumed a yellowish-brown color at 72 hr and by 96 hr was completely black. The black color of the "column" persisted throughout the study; no other visible gross changes were observed.

Histological Observations

24 hr post-tagging. The initial response consisted of a hemocytic infiltration of damaged muscle tissue adjacent to the pin. Immediately beneath the integument the pin had become partially encapsulated by several layers of hemocytes (Fig. 2). Associated with the hemocytes was a band of dark brown pigment presumed to be melanin similar to that described in the crayfish (Unestam and Nylund, 1972) and in insects (Salt, 1970). Interior from the integument, the hemocytes had not encapsulated the pin and the wound channel had a relatively smooth periphery of damaged muscle tissue. Foci of hemocytic encapsulations (nodules) were observed in the nearby muscle tissue.

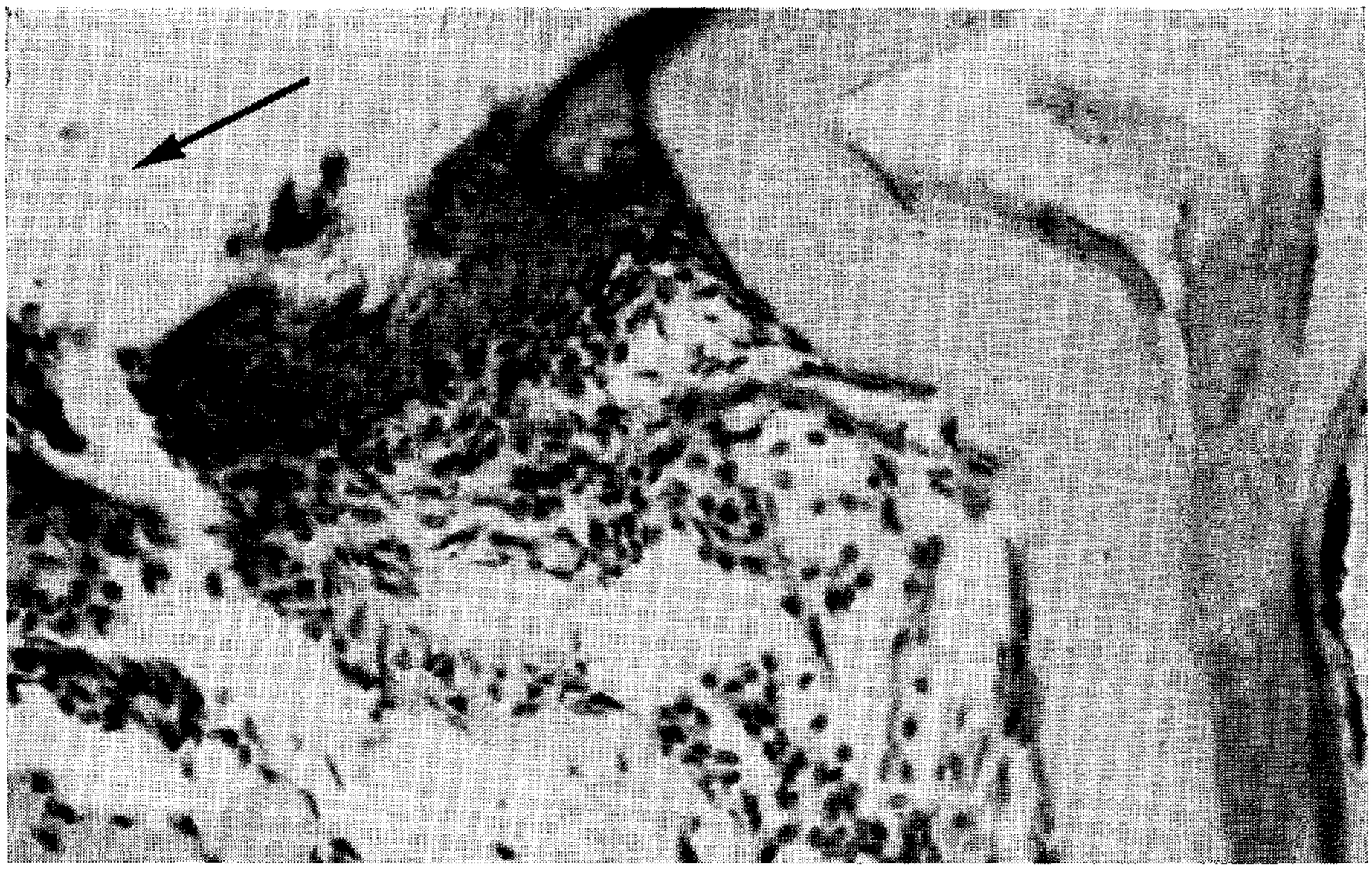

FIG. 2. Partial plating of the wound channel by hemocytes at 24 hr post-tagging; arrow indicates pin placement. Hematoxylin and eosin. ×320.

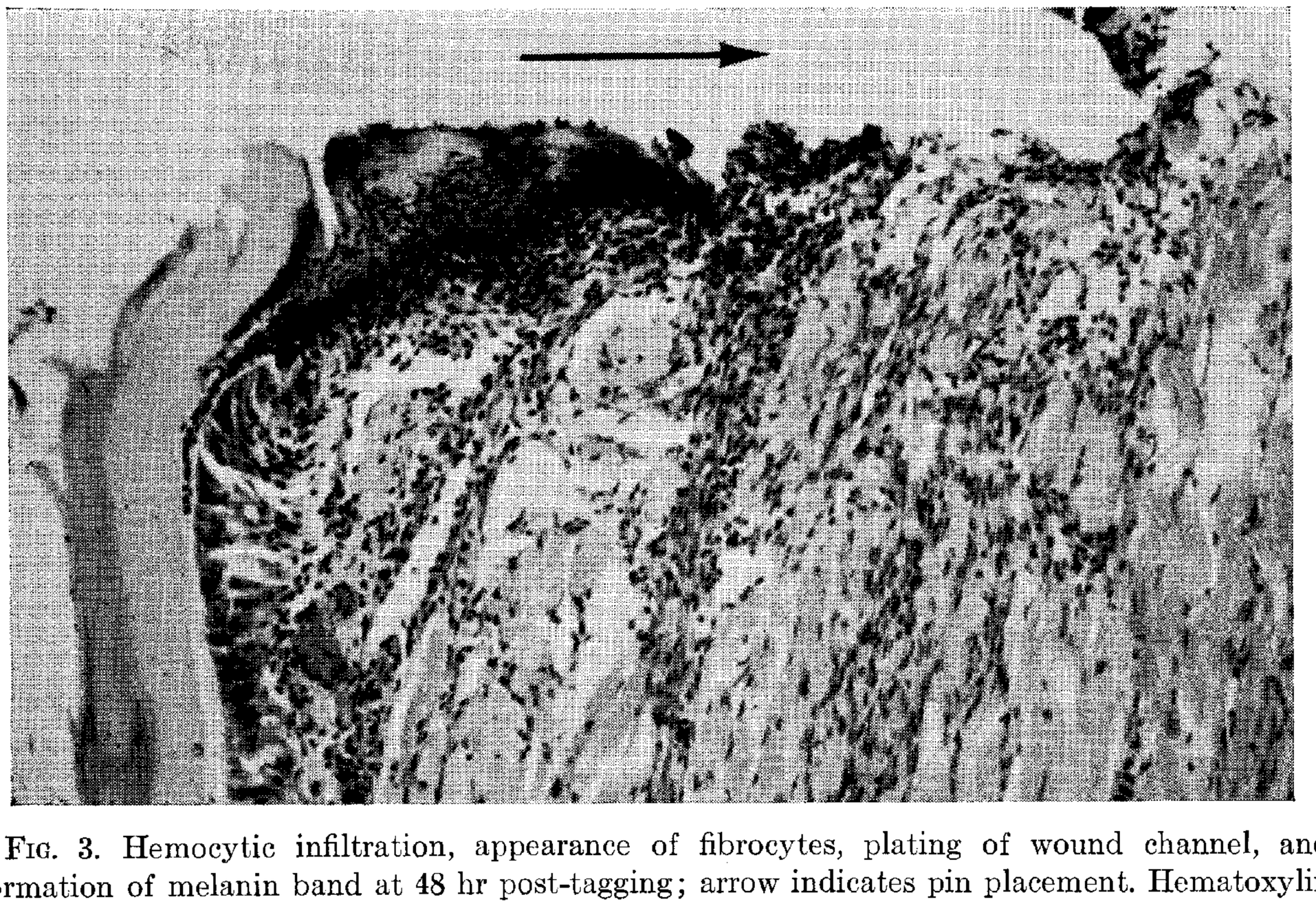

FIG. 3. Hemocytic infiltration, appearance of fibrocytes, plating of wound channel, and formation of melanin band at 48 hr post-tagging; arrow indicates pin placement. Hematoxylin and eosin. ×125.

48 hr post-tagging. The greatest hemocytic concentration was at the point of entrance and exit of the pin at 48 hr. The hemocytes circumscribing the pin were fusiform in configuration, and the concentric layers were banded by melanin. The cuticle at the opening of the wound had rounded edges and appeared to be involuting into the wound; however, there was no evidence of migration of the epidermis into the wound. Immediately adjacent to the wound the epidermis was composed of much taller columnar cells than that further from the wound or in nonwounded controls (Fig. 3).

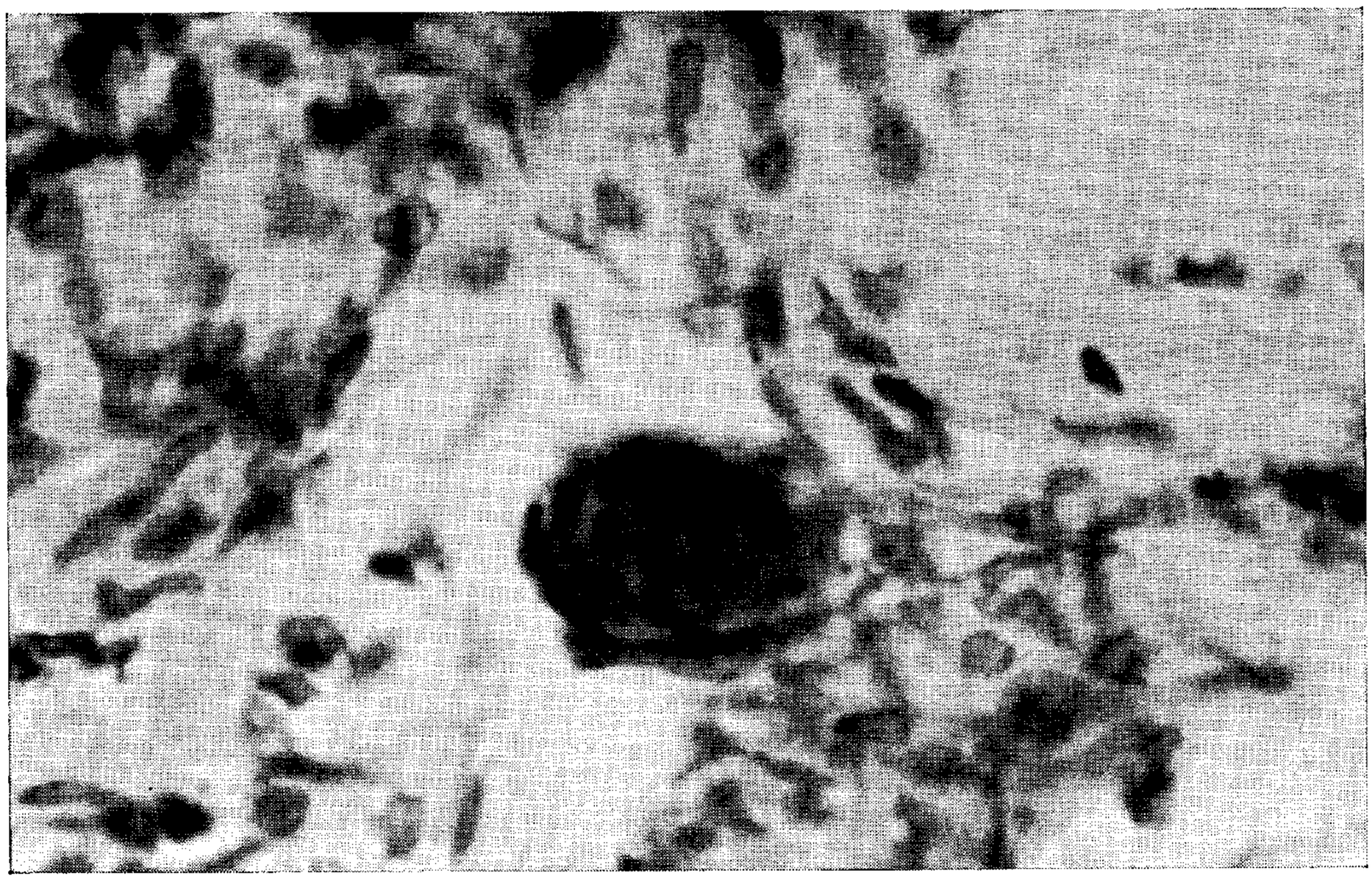

FIG. 4. A cellular encapsulation and melanin formation; 48 hr post-tagging. Hematoxylin and eosin. ×800.

Fibrocytes had appeared around the entrance and exit of the pin and associated with them were collagenlike fibers.

Somewhat interior to the body surface along the wound channel, circumscription of the pin by hemocytes was intermittent, some portions being covered with hemocytes while other areas adjacent to the pin remained relatively free. There was no evidence of phagocytic activity in hemocytes; however, phagocytosis may have been obscured by melanization in those areas where hemocytes had engaged in encapsulation. Lateral to the wound channel several small foci or nodules of necrotic tissues or foreign material introduced by the pin had been encapsulated by several layers of fusiform hemocytes and had become melanized (Fig. 4).

96 Hr post-tagging. Multiple layers of fusiform hemocytes were encapsulating the pin interior to the points of entrance and exist at 96 hr. Hemocytes in the layer nearest the pin had pyknotic nuclei while other hemocytes showed more advanced necrosis, including occasional cytolysis. The epidermis in the immediate vicinity of the wound opening appeared hypertrophic, possibly hyperplastic, and slightly hyperchromatic. Spindle-shaped fibrocytes had appeared at 96 hr around the pin and were associated with a fibrous collagenlike material. The epidermis had begun to migrate into the wound using the hemocytic network as a basal support (Fig. 5). The cuticle also appeared to be involuting but was probably being formed by the underlying epidermis.

192 Hr post-tagging. The entire pin was completely walled off by concentric layers of fusiform hemocytes at 192 hr (Fig. 6). The layers of hemocytes proximal to the pin had become necrotic with the periphery of the network composed entirely of eosinophilic cellular debris, while those hemocytes distal to the necrotic layer apparently remained active in circumscribing the pin and were characterized by a dark band of melanin (Fig. 7).

A thick matrix of fibroblasts and collagenlike fibers was deposited immediately basal to the involuting epidermis. The presence of fibers and the delineation of the wound by the fibrous complex was demonstrated in tissue sections stained with Mallory's triple stain (Fig. 8). Apparent

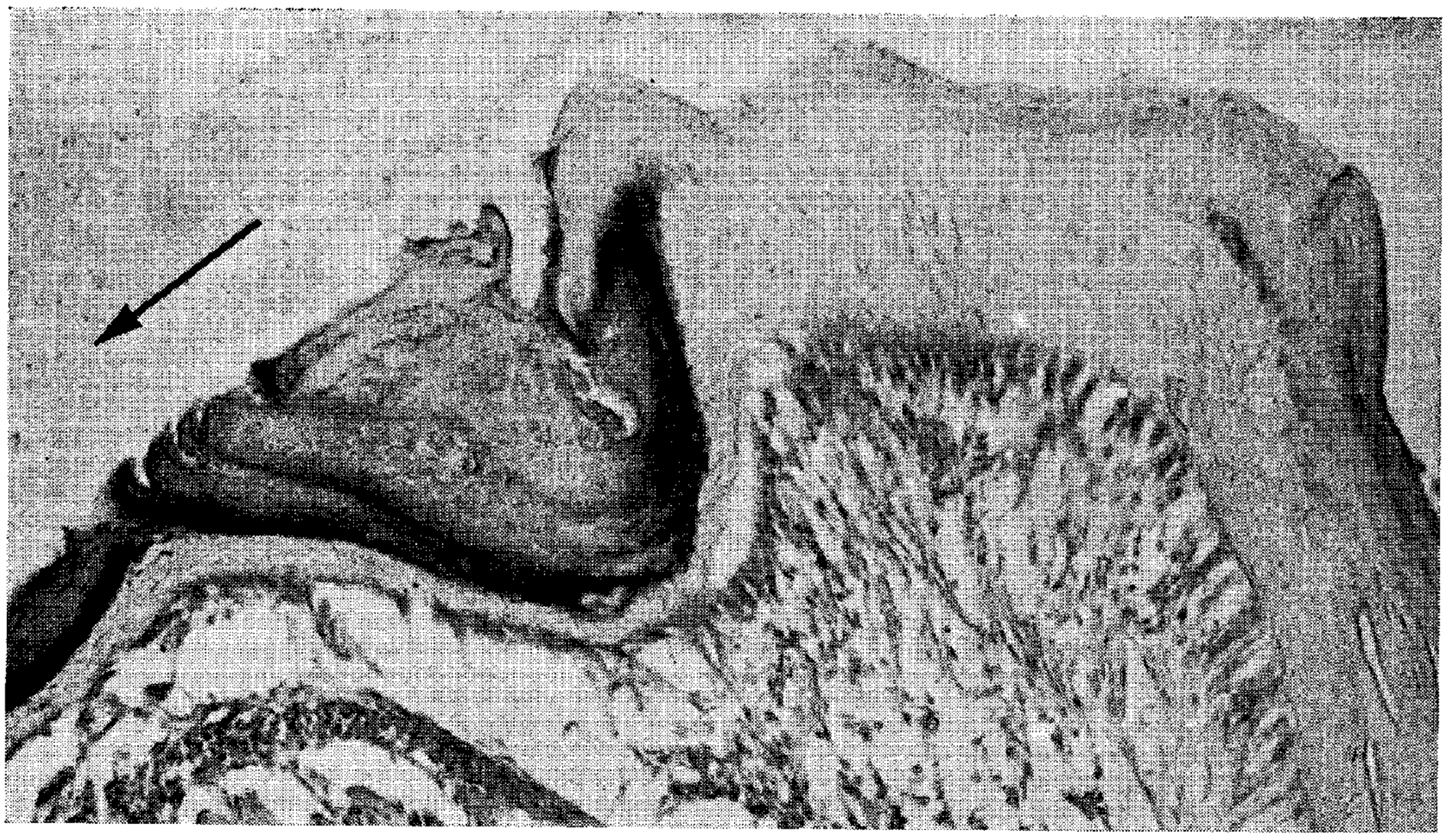

FIG. 5. The wound opening at 96 hr post-tagging; arrow indicates pin placement. The epidermis is migrating into the wound, and the exoskeleton appears to be involuting along the wound channel; note the four distinct bands of melanin. Hematoxylin and eosin. ×125.

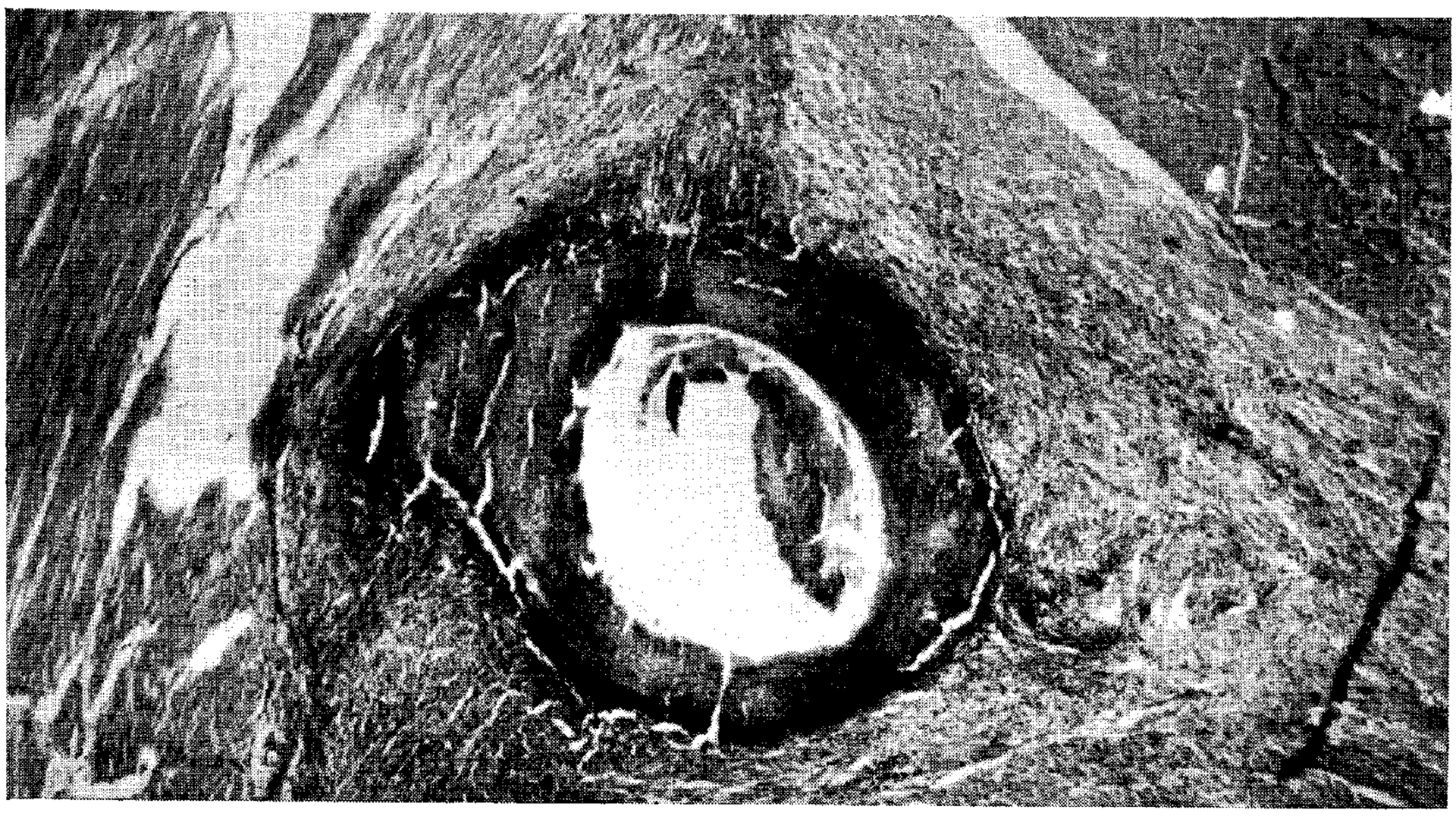

FIG. 6. The wound has been well defined by a thick matrix of fibrocytes and collagenlike fibers at 192 hr post-tagging (cross section of wound). The concentric layers of hemocytes around the pin are many cells thick. Hematoxylin and eosin. ×31.

phagocytosis by infiltrating hemocytes was occurring in many areas of damaged and necrotic muscle tissue surrounding the wound (Fig. 9). The material being phagocytized was probably nuclei from lysed hemocytes or muscle cells.

288 Hr post-tagging. At 288 hr the epidermis had migrated inward and had become continuous. Near the shrimp's midsagittal plane the epidermal epithelium was low columnar, while around the points of entrance and exit of the pin it appeared to be slightly hyperplastic. The epidermis nearest the wound opening was also somewhat hypertrophied, as it was larger than that seen in unwounded controls.

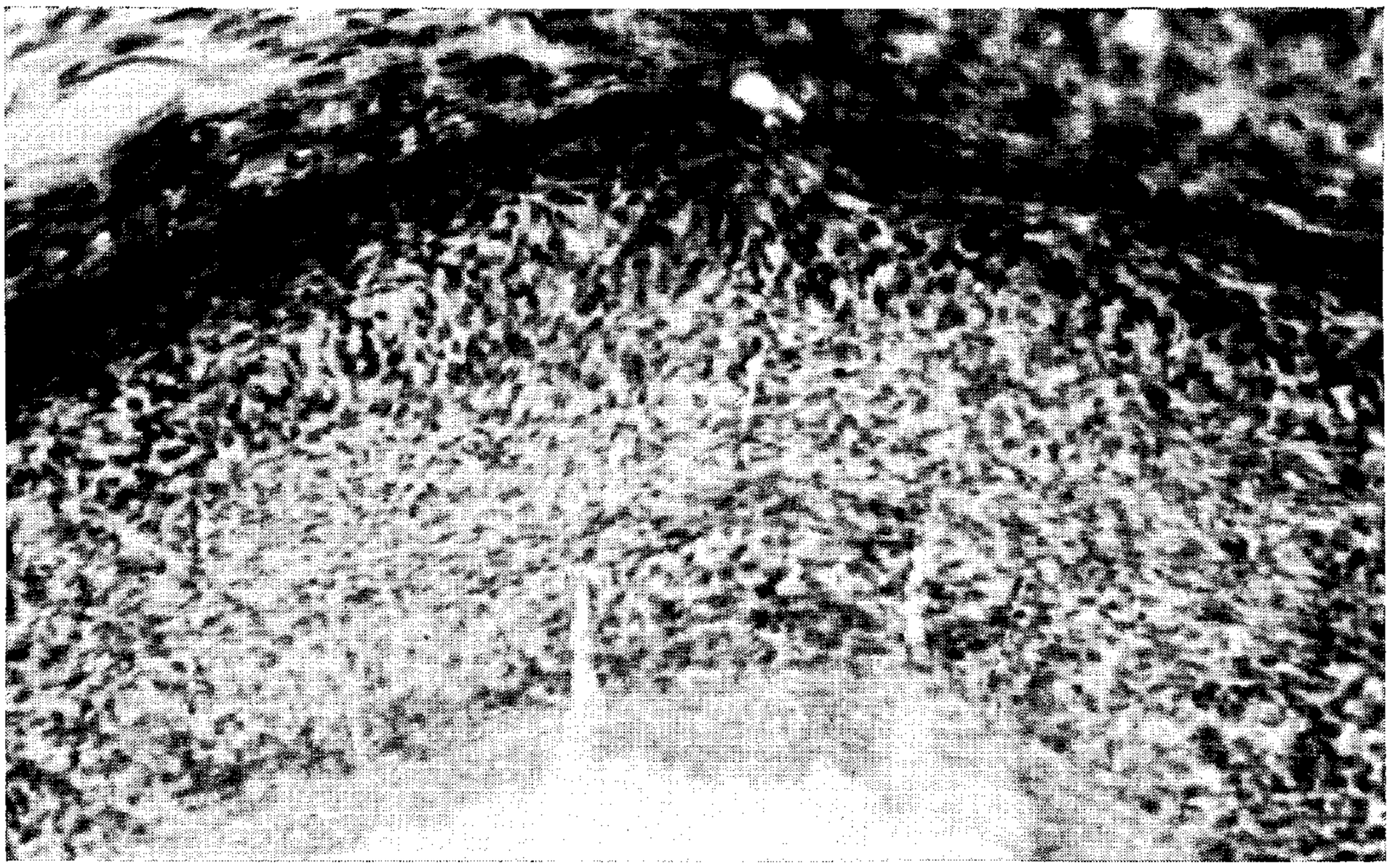

FIG. 7. The wall of the wound at 192 hr post-tagging. The outermost layers of hemocytes are necrotic while the innermost layer is actively engaged in producing the dark melanin band (cross section of the wound). Hematoxylin and eosin. ×320.

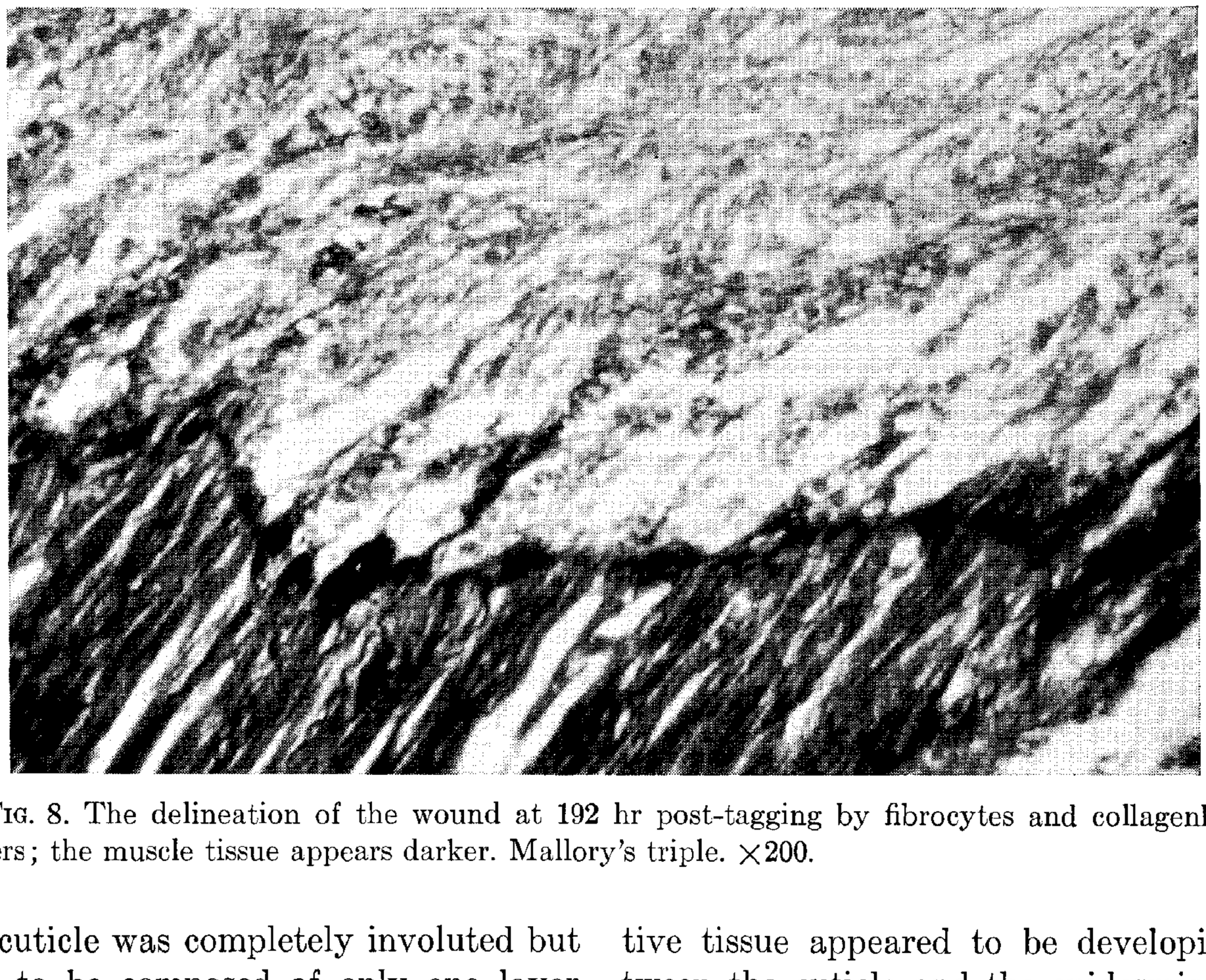

FIG. 8. The delineation of the wound at 192 hr post-tagging by fibrocytes and collagenlike fibers; the muscle tissue appears darker. Mallory's triple. ×200.

The cuticle was completely involuted but seemed to be composed of only one layer whereas the normal surface cuticle most often is composed of two layers, the outer epicuticle and the larger inner endocuticle (Dennell, 1960). A layer of loose connective tissue appeared to be developing between the cuticle and the epidermis in the wound channel, while basal to the epidermis a thick matrix of fibrocytes and collagenlike fibers had been depositied (Fig. 10). Also, basal to the epidermis, along the

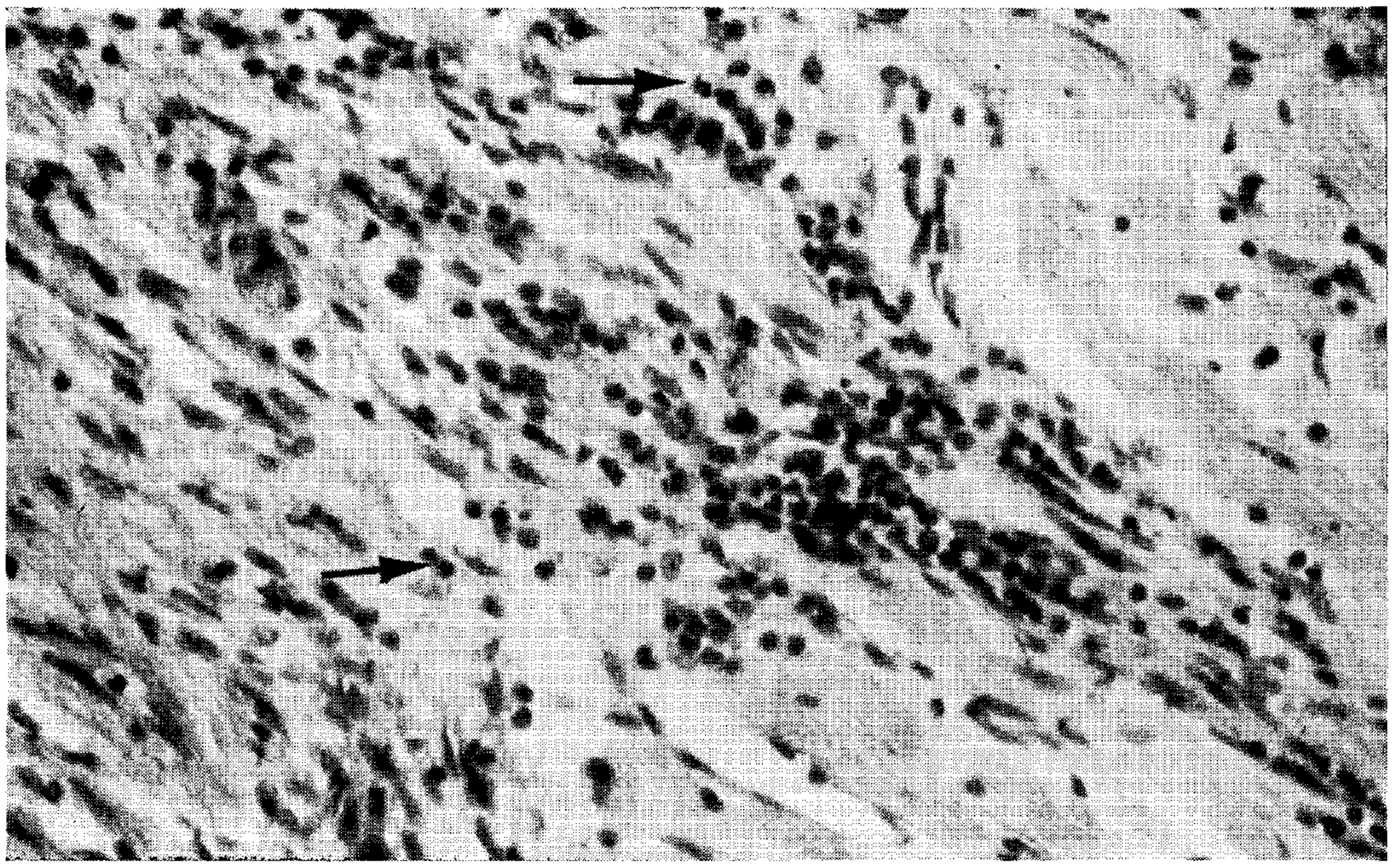

FIG. 9. The phagocytosis of foreign or necrotic material by hemocytes at 192 hr post-tagging. Hematoxylin and eosin. ×320.

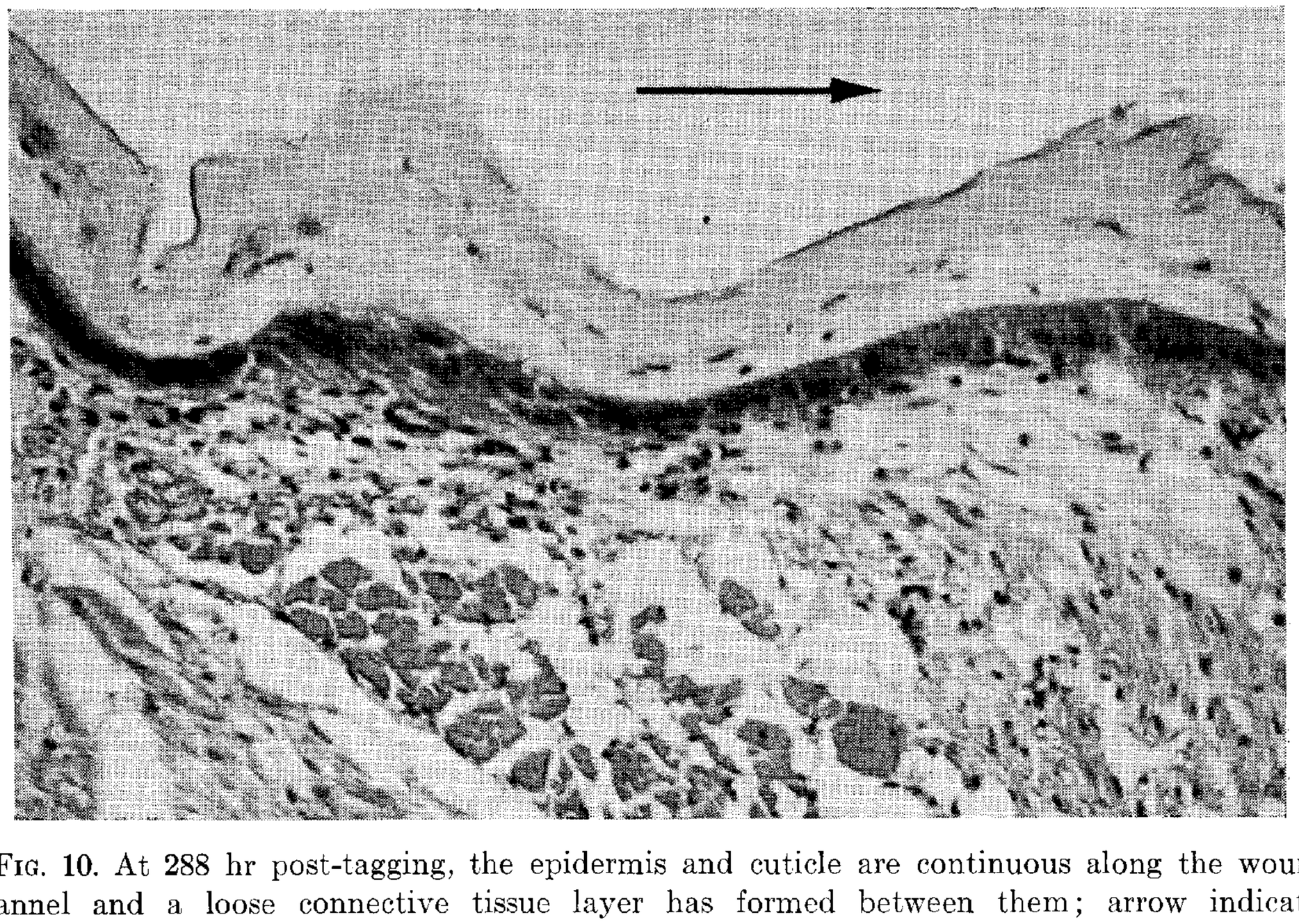

FIG. 10. At 288 hr post-tagging, the epidermis and cuticle are continuous along the wound channel and a loose connective tissue layer has formed between them; arrow indicates pin placement. Hematoxylin and eosin. ×200.

wound channel, large tegumentary or sebaceous-type glands (Dennell, 1960) were appearing.

Numerous nodules, some very large, were present in adjacent muscle tissue (Fig. 11). Many of these nodules were observed some distance away from the wound and were typically melanized with a whorled appearance (Fig. 12), and were probably induced by bacteria introduced into the tissue during insertion of the tag pin.

384 Hr post-tagging. The cuticle, now

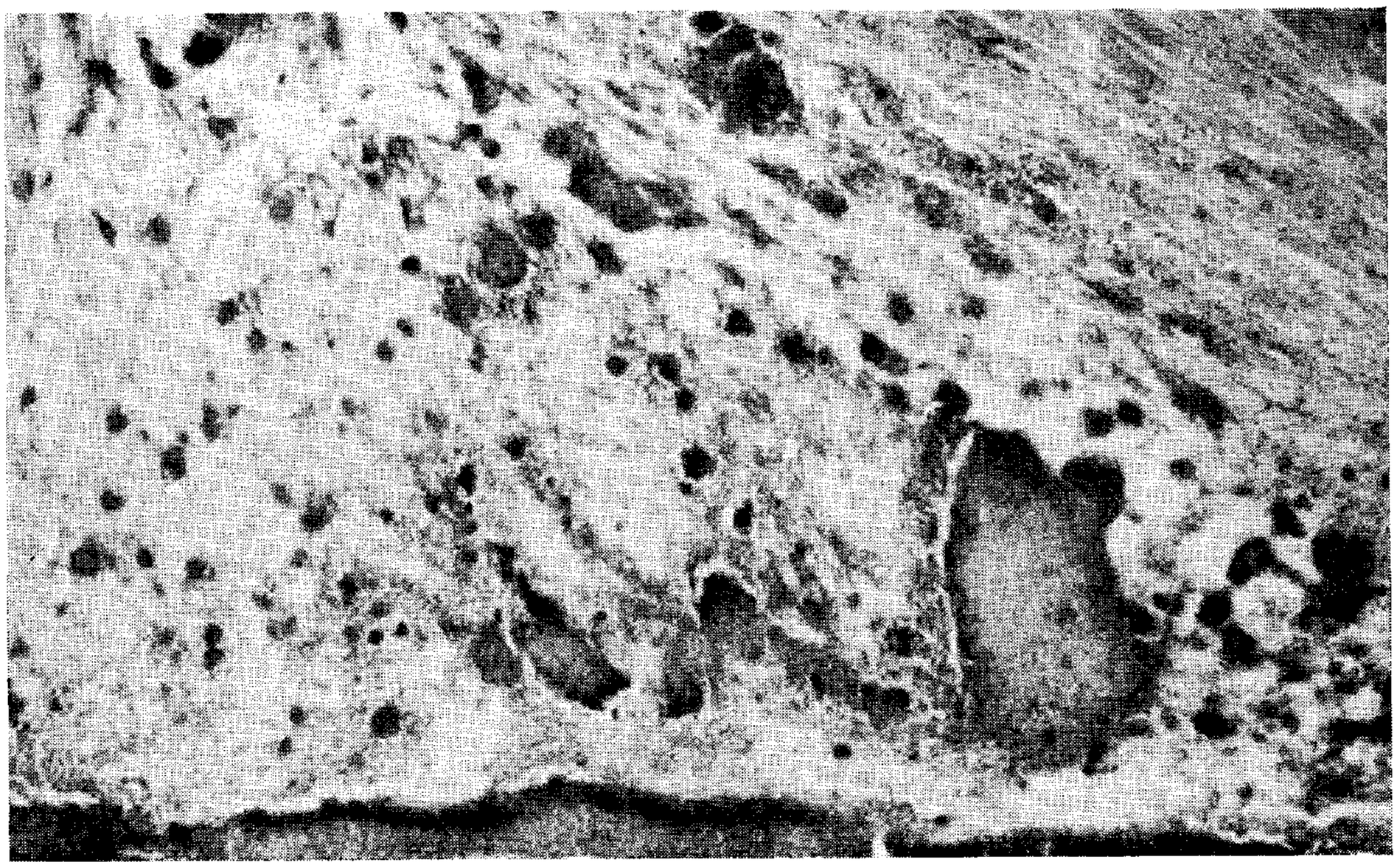

FIG. 11. Many foci of hemocytic encapsulations, several quite large, appeared along the wound channel at 288 hr post-tagging. Hematoxylin and eosin. ×50.

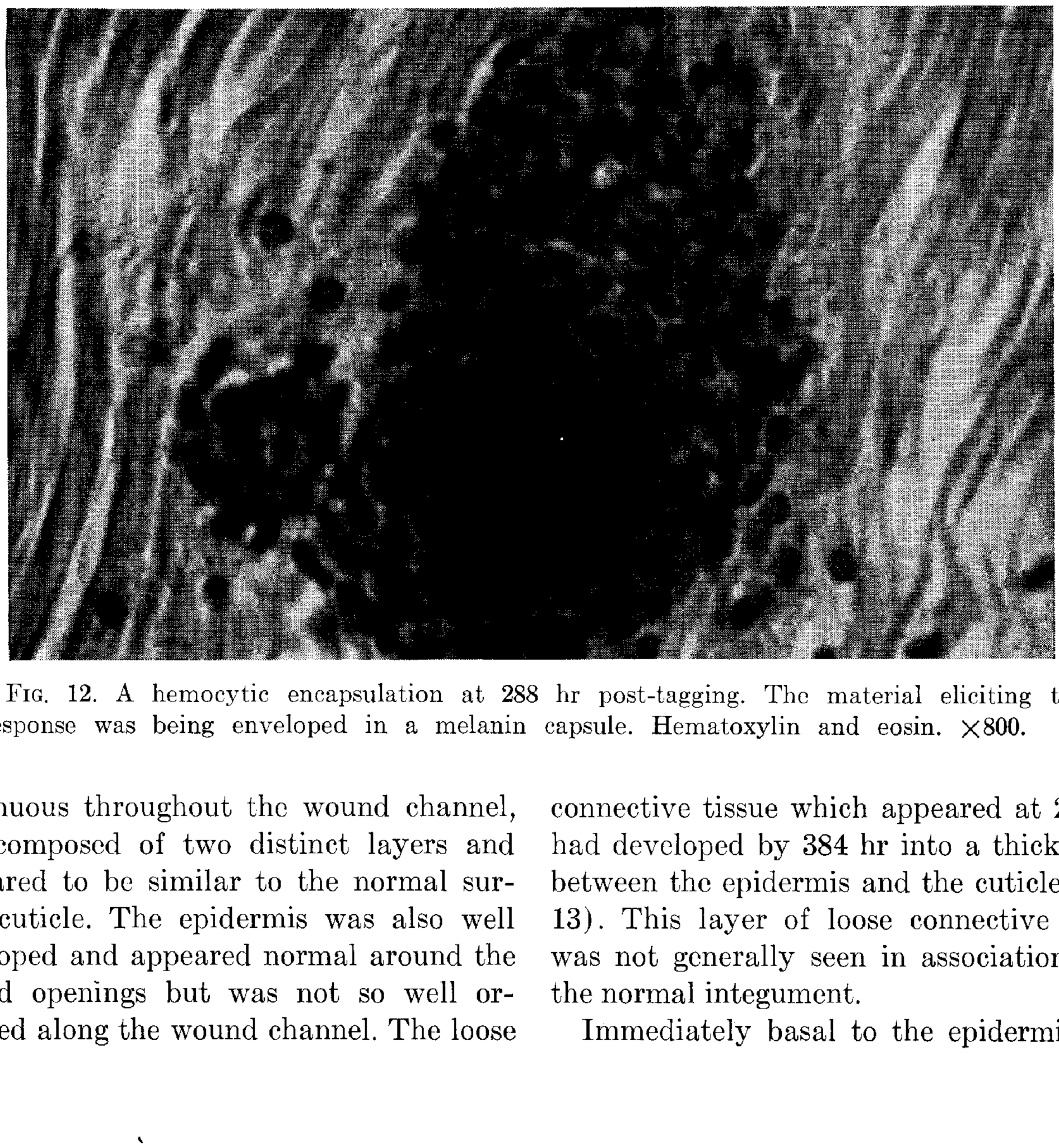

FIG. 12. A hemocytic encapsulation at 288 hr post-tagging. The material eliciting the response was being enveloped in a melanin capsule. Hematoxylin and eosin. ×800.

continuous throughout the wound channel, was composed of two distinct layers and appeared to be similar to the normal surface cuticle. The epidermis was also well developed and appeared normal around the wound openings but was not so well organized along the wound channel. The loose connective tissue which appeared at 288 hr had developed by 384 hr into a thick layer between the epidermis and the cuticle (Fig. 13). This layer of loose connective tissue was not generally seen in association with the normal integument.

Immediately basal to the epidermis was

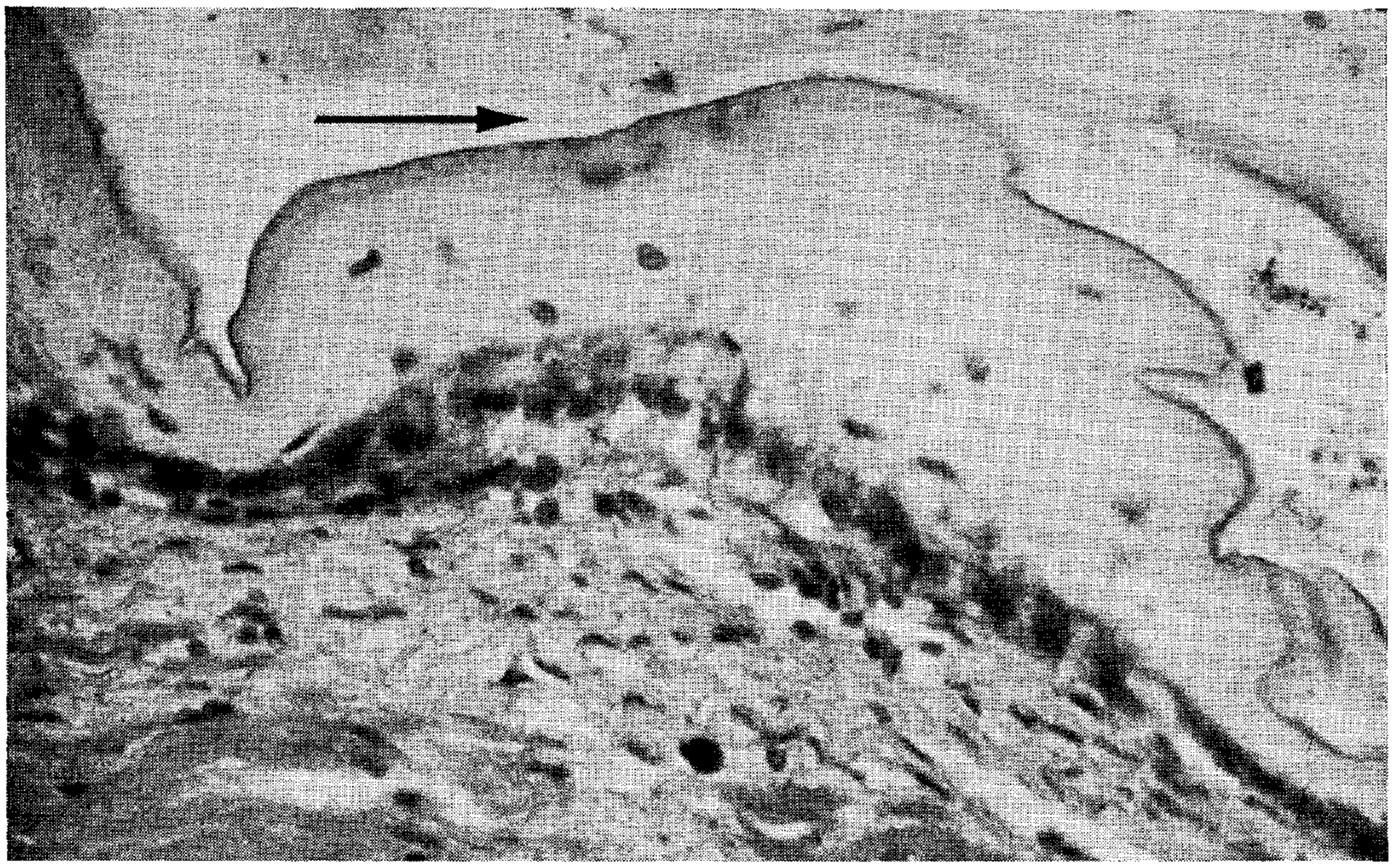

FIG. 13. The epidermis and cuticle were well developed and continuous at 384 hr post-tagging; the arrows indicate pin placement. Hematoxylin and eosin. ×320.

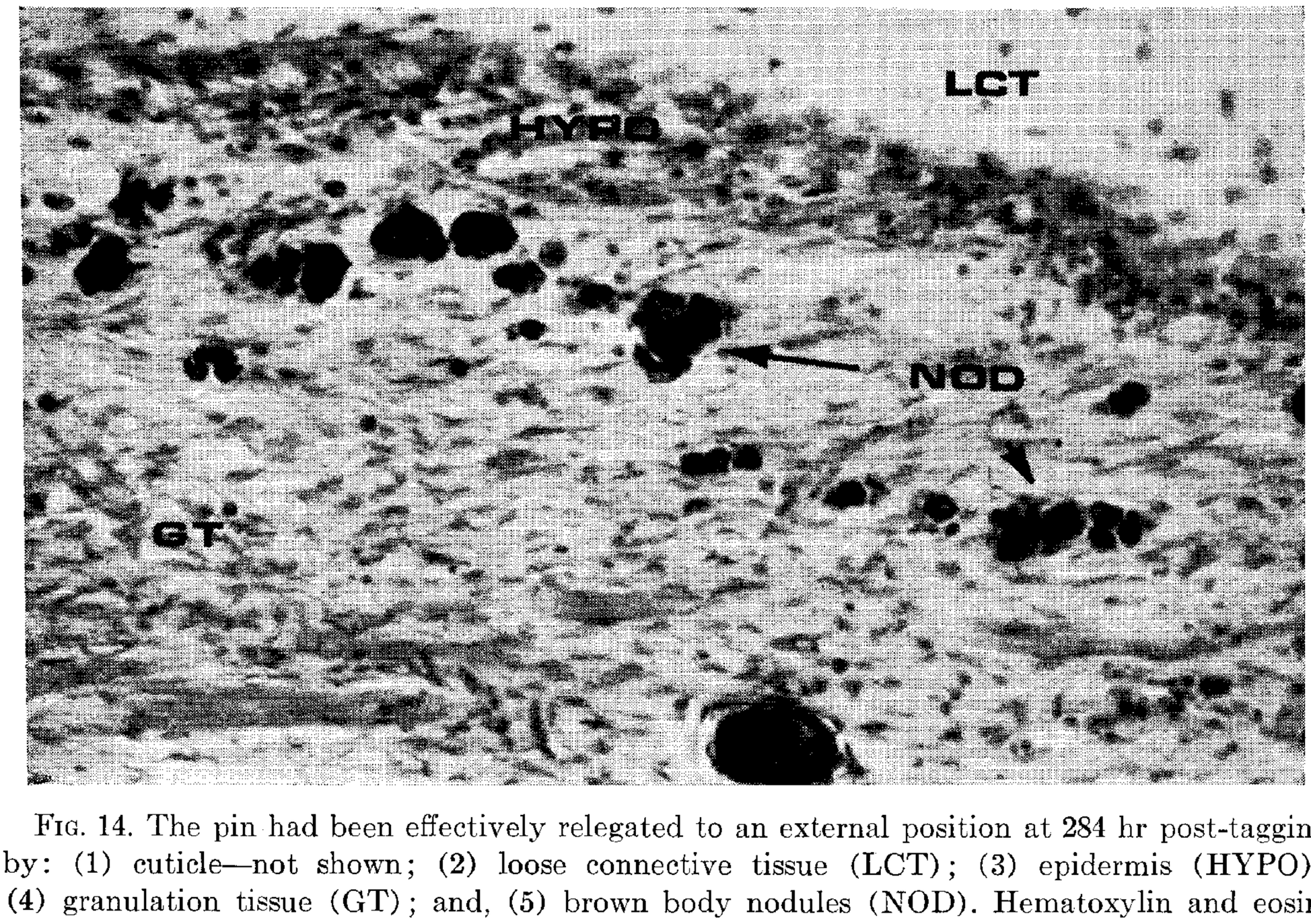

FIG. 14. The pin had been effectively relegated to an external position at 284 hr post-tagging by: (1) cuticle—not shown; (2) loose connective tissue (LCT); (3) epidermis (HYPO); (4) granulation tissue (GT); and, (5) brown body nodules (NOD). Hematoxylin and eosin. ×200.

a thick meshwork of fibroblasts and collagenlike fibers. Many rounded melanin nodules and several large tegumental glands were dispersed throughout this meshwork, but very few recognizable hemocytes remained in the wound area (Fig. 14). In many instances, the melanin nodules were composed of a primary wall with many smaller elements included (Fig. 15). The formation of melanin and the develop-

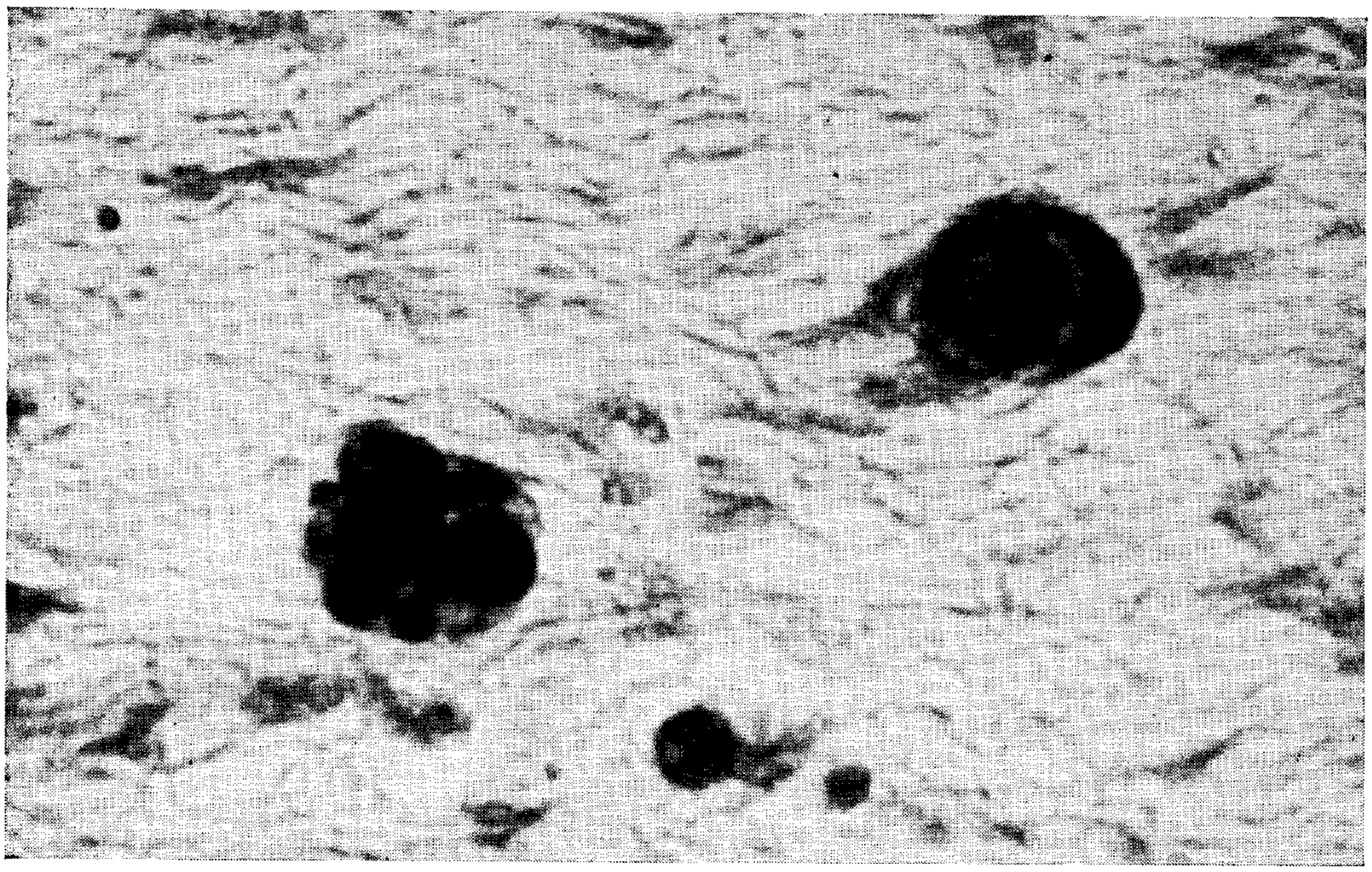

FIG. 15. The brown nodules dispersed throughout the granulation tissue basal to the epidermis at 384 hr were composed of many smaller elements with very few recognizable hemocytes in the area. Hematoxylin and eosin. ×800.

ment of the brown nodules probably imparted the dark color which was visible grossly at 72 hr and persisted throughout the study.

DISCUSSION

The wound repair mechanism in penaeid shrimp appears to be, in general, similar to that reported for several invertebrate groups (Sparks, 1973) and is not greatly unlike that seen in humans (Ross, 1969). After the initial response has been established, i.e., hemocytic and fibroblastic infiltration; however, the subsequent wound repair processes in penaeid shrimp resembles more closely that of insects (Salt, 1970). The most notable similarities between wound repair processes in penaeid shrimp and insects are in the formation of melanized nodules which result from hemocytic encapsulations and in the migration of the epidermis into the wound with the subsequent development of the cuticle to isolate the tag pin.

The exact composition of the brown substance that is apparently produced by penaeid shrimp hemocytes in response to environmental stress, disease, and injury is not known; however, Sindermann (1971) and Bang (1970) have referred to the brownish nodules or "brown bodies" found in decapod crustacea as chitin or chitinoid-like material. Although no specific chemical analysis of this material has been conducted for penaeid shrimp, we believe it to be melanin, similar to that described in crayfish (Unestam and Nylund, 1972) and in insects (Salt, 1970). It is also worthwhile to point out that, unlike wound repair in the oyster, *Crassostrea gigas*, described by Des Voigne and Sparks (1968), the wound repair observed here progresses from the external body surface internally along the wound channel. Furthermore, it is interesting to note that the integumental "tube" formed around the tag pin was not lost during normal ecdysis of test animals in this study.

In summary, the wound repair processes of penaeid shrimp following insertion of a stainless steel pin through the abdomen demonstrates the ability of the animal to respond to and repair a wound of some magnitude. The sequential histopathologi-

cal events in the wound repair process of penaeid shrimp are: (1) hemocytic infiltration; (2) encapsulation of the pin and of foreign or necrotic material by fusiform hemocytes and formation of melanin; (3) deposition of collagenlike fibers by fibrocytes; (4) phagocytosis of foreign or necrotic material by hemocytes; (5) migration of the epidermis into the wound; and, (6) the subsequent production of a cuticle by the migrating epidermis.

Acknowledgments

The authors are gratefully indebted to Mrs. Imogene Sanderson, who so diligently processed the specimens for histologic examination.

References

Bang, F. B. 1970. Disease mechanisms in crustacean and marine arthropods. *In* "A Symposium on Diseases of Fishes and Shellfishes" (S. F. Snieszko, ed.), pp. 383–404. Amer. Fish. Soc., Spec. Publ. 5.

Dennell, R. 1960. Integument and exoskeleton. *In* "The Physiology of Crustacea," Vol. I, "Metabolism and Growth" (T. H. Waterman, ed.). Academic Press, New York.

Des Voigne, D. M., and Sparks, A. K. 1968. The process of wound healing in the Pacific oyster, *Crassostrea gigas*. *J. Invertebr. Pathol.*, **12**, 53–65.

Fontaine, C. T. 1971. Exoskeletal intrusions: A wound repair process in penaeid shrimp. *J. Invertebr. Pathol.*, **18**, 301–303.

Krajian, A. A. 1940. "Histological Technique." Mosby, St. Louis, Missouri. 272 pp.

Lindner, M. J., and Anderson, W. W. 1956. Growth, migrations, spawning, and size distribution of shrimp, *Penaeus setiferus*. *U.S. Fish Wildl. Serv., Fish. Bull.*, **106**, 555–645.

Neal, R. A. 1969. Methods of marking shrimp. *FAO Fish. Rep.* No. 57, Vol. 3, pp. 1149–1165.

Ross, R. 1969. Wound healing. *Sci. Amer.*, **220**, 40–50.

Salt, G. 1970. "The Cellular Defense Reactions of Insects," Cambridge Monographs in Experimental Biology, No. 16. Cambridge Univ. Press, London and New York, 118 pp.

Sindermann, C. J. 1971. Internal defenses of crustacea: A review. *Fish. Bull.*, **69**, No. 3, pp. 455–489.

Sparks, A. K. 1973. "Invertebrate Pathology: Noncummunicable Diseases." Academic Press, New York.

Unestam, T., and Nylund, J. 1972. Blood reactions in vitro in crayfish against a fungal parasite, *Aphanomyces astaci*. *J. Invertebr. Pathol.*, **19**, 94–106.